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Effects of extracts of miswak and derum on proliferation of Balb/C 3T3 fibroblasts and viability of cariogenic bacteria

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Dates:
Accepted 27 July 2005

To cite this article:
Int J Dent Hygiene **4**, 2006; 62–66
Darmani H, Nusayr T, Al-Hiyasat AS.
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Abstract: *Objectives:* This study examined the effects of extracts of two chewing sticks on proliferation of fibroblasts and viability of cariogenic bacteria. *Methods:* Aqueous extracts of miswak (*Salvadora persica*; Arak tree) and derum (*Juglans regia*; walnut tree) were prepared and their effects investigated on growth of Balb/C 3T3 mouse fibroblasts by measuring the mitochondrial succinic dehydrogenase activity. Furthermore, the effects on the viability of various cariogenic bacteria (*Streptococcus mutans*, *Streptococcus salivarius*, *Lactobacillus casei* and *Actinomyces viscosus)* was also determined. *Results:* The data revealed that Balb/C 3T3 fibroblasts exposed to aqueous extracts of miswak or derum showed an increase in cell proliferation by 156% and 255%, respectively, in comparison with controls ($p<0.0001$). Furthermore, extracts from both miswak and derum had adverse effects on the growth of the cariogenic microorganisms, with derum having significantly greater antimicrobial effects than miswak and at much lower concentrations against all the bacteria tested. The most sensitive organisms were *A. viscosus*, followed by *S. mutans*, *S. salivarius*, with *L. casei* being the most resistant. *Conclusion:* The results show that aqueous extracts of miswak and derum enhance the growth of fibroblasts and inhibit the growth of cariogenic bacteria, with the derum extract showing greater activity than miswak.

Key words: antimicrobial; cariogenic bacteria; cell proliferation; chewing stick; derum; extract; fibroblasts; miswak

Introduction

For many centuries, different populations and cultures around the world have been using various tools, ranging from porcupine bones to chewing sticks to clean their teeth and gums (1, 2). The relative accessibility and the popularity of the chewing stick has made it a very cost effective agent for plaque control in different communities (1, 3–5). Although chewing sticks differ in their sources, effects and benefits the mechanical plaque-removing properties of chewing sticks are reported to be similar to that of conventional toothbrushes (6). The most widely used chewing stick is miswak, which is prepared from the roots or twigs of *Salvadora persica*, and is used in middle-eastern and eastern African cultures (4).

Various components of *Salvadora persica* and other related plants have been reported to have beneficial biological properties, including significant antibacterial and antifungal activity (7–9). Furthermore, extracts from these plants are reported to be effective against some periodontal pathogens and other bacteria that are important during development of dental plaque (10). It has therefore been proposed that these chewing sticks have anti-plaque effects and postulated that they may also affect the pathogenesis of periodontal disease by reducing the virulence of periodontopathic bacteria (11).

Derum is another natural 'chewing stick' obtained from the walnut tree (*Juglans regia*), and is used mainly by women in Saudi Arabia and some parts of India and Algeria as a toothbrush and as a cosmetic tool to colour their lips (12, 13). It has been reported to contain glycosides, resins, volatile oil, juglone, juglonic acid, phenolic acid and tannic acid (13). *Juglans regia* has been used in traditional medicine for antiparasitic, antihelmintic and repellant purposes (14). Like miswak, its extracts have shown a broad spectrum of antimicrobial activity (15). Interestingly, extracts of *J. regia* have been reported to inhibit *in vitro* growth, acid production and glucan-induced adherence of *Streptococcus mutans* (16). Furthermore, mouth rinsing with extracts of *J. regia* significantly reduced total streptococcal counts in salivary samples obtained up to 3 h after rinsing (16).

Despite the wide use of miswak and derum, information on the cytotoxic and antimicrobial effects of these chewing sticks are still scant. Thus, the present study aimed at investigating the effect of extracts of miswak and derum on the proliferation of Balb/C 3T3 mouse fibroblasts and on the growth of *S. mutans*, *Streptococcus salivarius*, *Lactobacillus casei* and *Actinomyces viscosus*.

Materials and methods

Extracts

Miswak or derum chewing sticks were cut into small pieces and ground to powder using a mill. Aqueous extracts were prepared by mixing 15 g of the powder of each chewing stick with 100 ml of sterile tissue culture grade distilled water and left for 48 h at 4°C. The mixture was then centrifuged at 2200 ***g*** for 10 min. The supernatants were sterilized using Millipore filters (0.20 μm pore size). The extracts were not stored but used immediately.

Cell culture and proliferation testing

Balb/C 3T3 mouse fibroblasts (Clone A31) (European Collection of Cell Culture, Salisburg, Wilts, UK) were used and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal bovine serum, 5% newborn calf serum, 100 unit ml^{-1} penicillin, 100 unit ml^{-1} streptomycin and 0.25 μg of amphotericin B at 37°C in a humidified incubator.

The effects of miswak and derum extracts on cell proliferation was assessed by the MTT (3{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide) assay. A 100 μl of cell suspension (1×10^5 cells ml^{-1}) was added to each well of a 96 well plate. Following this, the cells were incubated for 3 h at 37°C and 5% CO_2 to allow the cells to adhere. Aliquots (10 μl) of each of the extracts (seven dilutions) were added to the wells containing Balb/C fibroblasts (12 replicates). For controls, cells were exposed to an equivalent volume of distilled water. The cells were then incubated at 37°C with 5% CO_2 for 24 h. The MTT assay was performed in a sterile working area. MTT was prepared at a concentration of 3 mg ml^{-1} in phosphate-buffered saline and the reconstituted MTT was added in amounts equal to 10% of the culture media volume (10 μl). The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 3 h. After the incubation period, the resulting formazan crystals were dissolved by adding an amount of solubilization solution (acid isopropanol) equal to the original culture medium volume (100 μl) and the absorbance was measured at 580 nm using an ELISA plate reader (Titrek Multiskan plus EFIAB, Helsinki, Finland). DMEM without cells was used as a blank.

Cariogenic bacteria

Representative strains of four species of oral bacteria were selected: *S. mutans* (NCTC 10449), *S. salivarius* (NCTC 8606),

L. casei (NCTC 6375) and *A. viscosus* (NCTC 10951). The bacteria were cultured on blood agar base (Fluka Biochemica, Buchs, Switzerland) supplemented with 5% non-coagulant blood or tryptone soya broth.

Antibacterial effects of extracts of derum and miswak

Agar plate method

Aliquots (2 ml) of the diluted extract (20% v/v) were mixed with 20 ml of molten blood agar at 50°C and placed into sterile Petri dishes and allowed to set. The controls consisted of equivalent volumes of sterile distilled water. A known concentration of bacteria (100 μl aliquot) was inoculated onto the agar using a sterile glass spreader and the plates were then incubated at 37°C in an atmosphere of 5% CO_2 for 48 h. The number of visible colonies on these plates was then determined.

Minimum inhibitory concentration

The following concentrations of the extracts were prepared: 100% (neat; undiluted), 75%, 50%, 25%, 20%, 10%, 4%, 2% and 1% using TSB medium as the diluent. Aliquots of 2 ml of each concentration of extract were added to sterile screw-capped tubes and inoculated with 100 μl of a known concentration of a bacterial suspension and incubated at 37°C in an atmosphere of 5% CO_2 for 24 h. TSB without extract served as the control. The optical densities of the samples were then measured at 660 nm and compared with the corresponding standard curves (optical density versus number of bacteria per ml) prepared for each bacterial species, to determine the number of microorganisms per millilitre.

Data and statistical analysis

The effects of the extracts on cell proliferation was determined by measuring the succinate dehydrogenase activity (MTT assay) relative to controls (100% = no toxicity).

Data are expressed as mean ± SD. Differences between control and test groups were analysed using the Student's *t*-test. $P < 0.05$ was considered significant.

Results

Figure 1 shows the effects of aqueous extracts of miswak and derum on proliferation of Balb/C 3T3 fibroblasts. It can be seen that both miswak and derum extracts exerted significant increases in the proliferation of Balb/C 3T3 fibroblasts in comparison with the controls (100% viability). The increase in cell proliferation ranged from 119% to 156% with miswak while for the derum extract it ranged from 131% to >255%. Significant increases in cell proliferation were observed at all the concentrations of both chewing stick extracts ($P < 0.0005$).

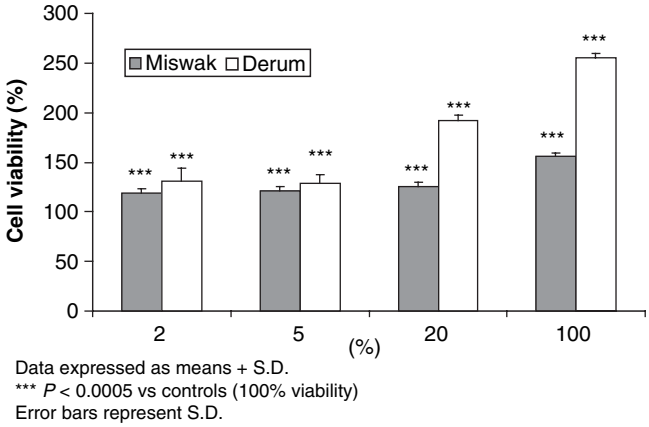

Fig 1. Effects of extracts of various concentrations (% v/v) of miswak and derum on proliferation of Balb/C 3T3 fibroblasts.

Figure 2 shows the percentage viability of *S. mutans*, *S. salivarius*, *L. casei* and *A. viscosus* cultivated on agar containing extracts of miswak and derum. The control for each organism was taken to represent 100% viability. Significant reductions were observed in the viability of *S. mutans* ($P < 0.001$ and $P < 0.0005$ for miswak and derum, respectively), *S. salivarius* ($P < 0.05$ for both miswak and derum), *A. viscosus* ($P < 0.05$ and $P < 0.001$ for miswak and derum, respectively) in comparison with the controls, but not in the viability of *L. casei*, with the extracts from both miswak and derum. The most sensitive organism was *A. viscosus*, followed by *S. mutans* and *S. salivarius*. Furthermore, the extract of derum exerted greater effects on viability of the microorganisms tested when compared with miswak.

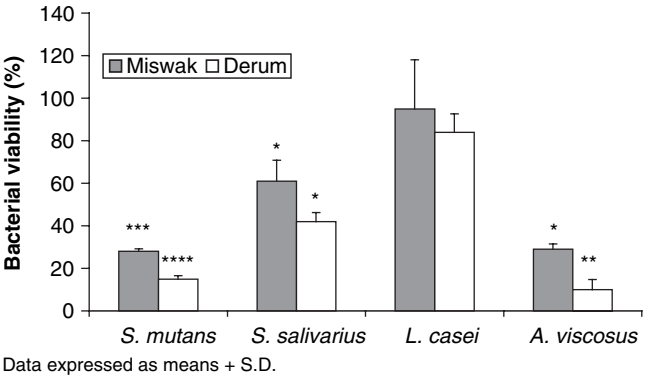

Fig 2. Effects of extracts of miswak and derum on viability of cariogenic bacteria.

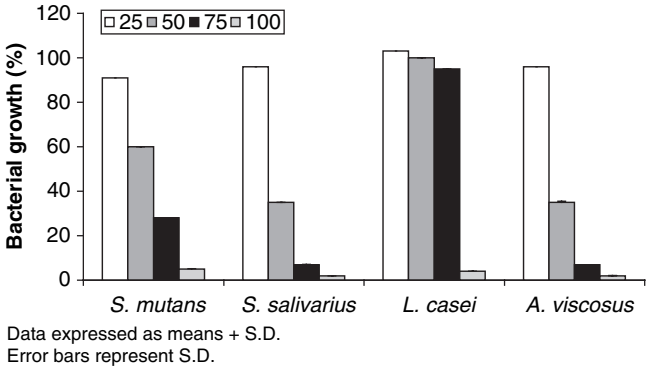

Fig 3. Effects of various concentrations (% v/v) of an extract of miswak on growth of cariogenic bacteria.

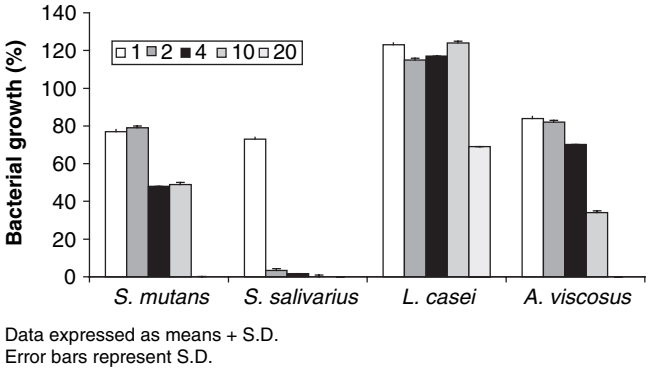

Fig 4. Effects of various concentrations (% v/v) of an extract of derum on growth of cariogenic bacteria.

The minimum inhibitory concentration (MIC) was regarded as the lowest concentration of the extract which inhibits the growth of bacteria, which was considered in this study to be the statistically significant inhibitory effect exhibited by the extracts on the growth of the cariogenic bacteria. Figures 3 and 4 show the effects of different concentrations of extracts of miswak and derum on the growth of the various cariogenic bacteria. Figure 3 shows that extracts of miswak exerted a significant reduction in the growth of *S. mutans*, *S. salivarius*, *A. viscosus* and the MIC for these organisms was 50% (v/v). However, derum extract exerted a greater inhibitory effect on the growth of *S. mutans*, *S. salivarius* and *A. viscosus* and the MIC for these organisms was 1%, 2% and 1% (v/v) respectively. Figure 3 shows that miswak exerted a significant reduction in the growth of *L. casei* only at a concentration of 100% while derum extract exerted a greater inhibitory effect on this microorganism with an MIC of 20% (v/v).

Discussion

Aqueous extracts from both chewing sticks exerted a proliferative effect on Balb/C 3T3 fibroblasts in a dose-dependent manner. However, derum extract had a greater effect on the cells than miswak even at the lowest dilution tested. This is probably because of the different chemical constituents that are present in each chewing stick. The results suggest that derum had more components that had leached out of it, or its components were more soluble in water than those of miswak. Furthermore, the types of components present in derum might have a greater capacity for inducing cell proliferation than those of miswak. Interestingly, it has been reported that because of the presence of juglone and phenolic acid, the continuous use of derum has a tendency to produce neoplastic changes in the mucosa (13). Furthermore, juglone has been reported to have tumour promoting activity in mice (17).

The increased cell proliferation, observed with the miswak extracts, are in line with previous reports which showed that miswak has gum stimulating effects (18). Thus, the results of the current study suggest that extracts of miswak and derum increased the proliferation of fibroblasts. It is well known that cellular proliferation plays an important role in both physiological and pathological processes. Cell proliferation is regulated by steroid hormones and growth factors and during wound healing the process is stringently controlled. Little is known about the components contained in the miswak extract that may enhance cell proliferation and further research on this effect is needed. Derum, on the other hand, is known to have possible carcinogenic effects. Indeed, as mentioned earlier, daily use of juglone-containing preparations of walnut bark is tied to an increased occurrence of cancer of the tongue and leukoplakia of the lips (17). Furthermore, juglone initiated skin carcinomas and papillomas in Sencar mice when applied dermally (19). No epidemiological studies or case reports investigating the association of exposure to juglone and cancer risk in humans were identified in the available literature. Juglone in the presence of a tumour initiator [like 7,12-dimethylbenz[a]anthracene (DMBA)] initiated skin carcinomas and papillomas in Sencar mice when applied dermally. Tumour incidence and tumour multiplicity were both dose-dependent.

The results of the current study also indicate that both miswak and derum extracts had significant inhibitory effects on the growth of the different cariogenic bacteria tested. Furthermore, a greater inhibitory effect was obtained with the derum extract than with the miswak extract against all microorganisms tested. The most sensitive organisms were *A. viscosus*, followed by *S. mutans*, *S. salivarius*, with *L. casei* being the most resistant.

Our results are in agreement with other *in vitro* studies which have shown that *Salvadora persica* extracts inhibit growth of various oral aerobic and anaerobic bacteria (7, 8) Almas and Al-Bagieh (20) found that aqueous extracts of

Salvadora persica bark, the pulp as well as the whole miswak, were effective against various bacteria including *S. mutans*. Dorner (21) speculated that the high amount of NaCl, KCl, trimethylamine and sulphur-containing organic substances (salvadourea and salvadorine) in miswak might somehow be responsible for the observed antibacterial and gum-stimulating effects (18). Furthermore, a recent study by Darout *et al.* (6) showed that aqueous miswak extracts contained potential antimicrobial anionic components in addition to chloride and sulphate, which were thiocyanate and nitrate. They hypothesized that thiocyanate leaching out from miswak, while in the oral cavity, may lead to an elevated level of salivary thiocyanate. This, in turn, may enhance the efficacy of the salivary hydrogen peroxide–peroxidase-thiocyanate system, a known antimicrobial component of human saliva (22). This may partly explain the observation that adult Sudanese miswak users had significantly lower numbers of cariogenic bacteria in their saliva while the matched toothbrush users demonstrated lower salivary levels of periodontic pathogens (6).

As stated previously, very little information exists about the antimicrobial properties of derum. Jagtap and Karkera (16) reported that aqueous and alcoholic extracts of *J. regia* inhibited the *in vitro* growth, adherence, acid production and glucan-induced adherence of *S. mutans*. In agreement, the results of the current study indicate that derum extract inhibited the growth of all the microorganisms tested more effectively than miswak extract. Indeed much lower MICs were obtained for extracts of derum against *S. mutans*, *A. viscosus*, and *S. salivarius* (MIC: 1%, 1% and 2% v/v, respectively) while for miswak the MICs were much higher (50% v/v). Furthermore, extracts of derum significantly inhibited the growth of *L. casei* at a concentration of 20% while the MIC with miswak was 100% (v/v).

Finally, under the conditions of the study we can conclude that derum has a greater effect in increasing the proliferation of Balb/C 3T3 fibroblasts. Furthermore derum extract exerted significantly greater antimicrobial effects than miswak at much lower concentrations against the cariogenic bacteria tested.

Acknowledgements

This work was supported by a grant from the Deanship of Research at Jordan University of Science and Technology and was part of an MSc thesis.

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